Reference: *Biol. Bull.*, 141: 122-129. (August, 1971)

UPTAKE AND RELEASE OF FREE AMINO ACIDS BY STARFISHES[1]

JOHN CARRUTHERS FERGUSON

Department of Biology, Florida Presbyterian College, St. Petersburg, Florida 33733

In the last few years a considerable quantity of data has been accumulated to indicate that many species of marine invertebrates possess the ability to remove dissolved amino acids and other nutritional compounds directly from sea water, even when these materials are present in very low concentrations. This phenomenon was first clearly demonstrated by Stephens and Schinske in 1961 on representatives from 10 phyla, including Echinodermata. Stephens and his colleagues have continued to examine several aspects of the uptake of organic compounds by various marine species, including a coral, sipunculid, annelids, and brittlestars. Their observations are presented in a series of reports (Stephens, 1962, 1963, 1964; Stephens and Virkar, 1966; Virkar, 1966).

My own work (Ferguson, 1963, 1967a, 1967b, 1968a; 1969, 1970) has confirmed the ability of several starfishes to take up dissolved organic compounds from sea water, and revealed a number of interesting features of the process. Most important, however, is the observation that while in specific instances oral (and even rectal) ingestion of dissolved nutrients may occur, uptake and utilization of these compounds is of primary importance only to the epidermis. Indeed, evidence has been accumulated which indicates that the epidermis is to a large extent functionally isolated from internal reservoirs of nutrients obtained through normal feeding activity, and must sustain itself primarily from the free organic compounds it can scavenge from the external media. Thus, epidermal uptake would complement feeding activity. It is a continuous process as opposed to the intermittent accumulation of foodstuffs in feeding, and is mainly significant to only a very small volume of cells with a very large exposed surface area. Such uptake however, may be essential to the survival of these cells.

The concept that marine animals can derive net benefit from dissolved organic compounds has not, however, achieved universal acceptance. Critics have noted the extremely low concentrations of dissolved amino acids found in "normal" sea waters (usually less than 1 micromolar total; *cf.,* Chau and Riley, 1966; Siegel and Degens, 1966; Webb and Wood, 1967), although reliable data is not available on the concentrations in the microenvironments of individual species. It has not been possible to determine how much energy the epidermal cells need expend to take up and retain nutrients from such dilute solutions. It is possible that the uptake mechanism could be coupled with other essential processes in the cells, metabolic alteration of the substrate, or physical adsorptive phenomena. For these reasons, the additional energy costs of transport to metabolism could be almost negligible. In any case, concern for energy budgets in the transport system does not at present appear to be a reasonable restriction to further inquiry.

[1] Supported by NSF grant GB-14649 to Florida Presbyterian College and NSF Grant GB-5531 to the Friday Harbor Laboratories of the University of Washington.

The tracer techniques generally used to demonstrate uptake by a particular organism do not provide reliable information on the dissolved nutrients released back into the environment, and thus the net benefit from the uptake. This last point has been noted in a study by Johannes, Coward, and Webb (1969) on the commensal flatworm, *Bdelloura*. With the use of ion-exchange methods of analysis these workers observed that specimens of this species released into the media approximately 3 times as much amino acid as they took up. It should be apparent, however, that even if this observation were generalized to the great many species for which uptake of dissolved nutrients have been demonstrated, it would not lessen the importance of such uptake in the nutrition of specific tissues, such as the epidermis. Indeed, the nutrition of this tissue would be benefited if there were excretion of nutrients into its close proximity by other portions of the body! There is as yet no reason to believe that the observations of Johannes *et al.* (1969) should be broadly generalized.

Certainly many studies need to be completed on the uptake and release of free organic compounds by various species, and evaluation made of the net benefit derived from these nutrients. Such studies are only now becoming possible as new analytical techniques are developed. In the present investigation use has been made of gas-liquid chromatography to obtain initial information on the net uptake and release of amino acids by several species of starfishes.

The author is indebted to the Friday Harbor Laboratories of the University of Washington for providing facilities for this study.

Materials and Methods

Ten species of starfishes from the Puget Sound area were used in the study. Represented were the following: *Pteraster tesselatus, Mediaster aequalis, Patiria miniata, Pisaster ochraceus, Dermasterias imbricata, Solaster stimpsoni, Stylasterias ferreri, Evasterias troschelii, Pycnopodia helianthoides,* and *Henricia leviuscula.* For the experiments, each specimen was placed in 750 ml of medium in a glass vessel partially submerged in fresh running sea water (11° C) on a sea table. The media consisted of freshly filtered (Millipore 0.45 micron) sea water to which known quantities of various pure amino acids were added. Twenty-five ml aliquots of the media were withdrawn at the beginning of the experiment and after 6 hours. These were placed in serum vials together with a measured quantity of β-alanine as an internal standard. The vials were frozen, lyophilized, and subsequently extracted with a 2 ml and a 0.5 ml portion of 90% ethanol containing 5% 1 *N* HCl. Solution of the amino acids was enhanced by placing the vials in an ultrasonic cleaning bath for several minutes. After centrifuging, the supernatant was slowly placed through a 1 × 14 cm column one-half full of regenerated Amberlite IR-120 H resin. The resin was then rinsed with 10 to 12 ml of double distilled water placed through the column in several aliquotes. The amino acids were eluted with 10 ml of repurified 7 *N* ammonium hydroxide. One ml of the eluate was placed in a small screwcap tube and dried under nitrogen in a sand bath at 100° C.

The amino acid samples were then quantitatively converted to N-triflouro-acetyl n-butyl esters by the direct esterification method of Roach and Gehrke (1969). Separation was achieved by injecting approximately 7 microliters of

the sample into a 4 foot glass column containing 0.65% ethylene glycol adipate on Chromosorb W in a Beckman GC-45 temperature programed gas chromatograph. This instrument was equipped with hydrogen flame ionization detectors and a disc integrator on the recorder. Instrument settings included 80° C initial temperature and a 32 minute 135° C rise after a 20% hold. Peaks were quantified by comparing their areas to that of the internal standard.

This method was sensitive to approximately 0.5 micromolar concentrations in the media (0.0125 micromoles per 25 ml sample) of the following amino acids: *l*-alanine, *l*-valine, glycine, *l*-isoleucine, *l*-leucine, *l*-proline, *l*-threonine, *l*-serine, *l*-cysteine, *l*-methionine, *l*-hydroxyproline, *l*-phenylalanine, *l*-aspartic acid, *l*-glutamic acid, *l*-tyrosine, *l*-lysine, and *l*-tryptophan. Sensitivity was primarily limited by the practical extent to which the various reagents used could be purified—especially the ion exchange resin.

TABLE I

Net change in amino acid content of media after 6 hours exposure to specimens (Initial concentration = 0.050 mM/l L-alanine)

Specimen	Wet wt. (g)	Volume medium (ml)	Initial *L*-alanine (μM)	Final *L*-alanine (μM)	Final total other a.a. (μM)	Principal a.a. released
Pteraster	178	750	37.5	0.5	0.5	GLY
Pteraster	249	750	37.5	tr.	0.0	
Pteraster	218	750	37.5	0.5	1.0	Gly, SER
Mediaster	49	750	37.5	20.0	0.5	GLY
Mediaster	19	750	37.5	29.5	2.0	GLY, SER, CYSH
Mediaster	73	750	37.5	15.5	1.0	SER
Patiria	184	750	37.5	7.0	0.0	
Patiria	146	750	37.5	15.5	1.0	GLY, GLU, TYR
Patiria	210	750	37.5	9.5	1.5	GLY, SER
Pisaster	361	750	37.5	4.0	1.5	GLY
Pisaster	381	750	37.5	3.0	0.5	GLY
Dermasterias	340	750	37.5	12.0	3.5	GLY, SER, ILEU, VAL
Solaster	272	750	37.5	0.5	5.5	MET, GLY
Stylasterias	300	750	37.5	tr.	2.0	GLY
Evasterias	810	1000	50.0	0.5	2.5	GLY
Pycnopodia	88	750	37.5	0.5	1.0	GLY, SER

RESULTS

Three main series of experiments were carried out. In the first of these an attempt was made to see if there is any net release of amino acids by starfishes into their media under relatively normal conditions. A low concentration (0.05 mM/l) of L-alanine was included in the media to verify that uptake mechanisms were functioning. The results are shown in Table I. In most cases only trace quantities (too low to be accurately measured) of glycine and occasionally other amino acids were detected in the media at the end of the 6-hour experimental period. The larger specimens took up most of the alanine present, and the smaller ones a large proportion of it.

In the second series, the starfishes were exposed to a mixture of amino acids, each initially present in a 0.05 mM/l concentration, to see whether the net effect

TABLE II

Net change in content of 12 amino acids in media after 6 hours exposure to specimens (Initial concentration = 0.05 mM/l; Volume, 750 ml)*

	Initial quantity	Quantity present after 6 hours					
Specimen wet. wt.		*Pteraster* 208 g	*Pteraster* 283 g	*Pteraster* 267 g	*Patiria* 191 g	*Patiria* 199 g	*Patiria* 174 g
Amino acid	(μM)	(μM)	(μM)	(μM)	(μM)	(μM)	(μM)
L-alanine	37.5	28.0	14.0	4.0	26.5	24.5	31.5
L-valine	37.5	28.5	18.0	5.0	26.0	26.0	35.5
glycine	37.5	35.5	40.5	48.5	26.5	23.0	27.0
L-*proline*	37.5	30.0	26.5	5.0	24.5	26.0	35.5
L-threonine	37.5	27.5	25.0	13.0	25.0	25.0	33.0
L-serine	37.5	22.5	21.5	8.5	23.0	22.0	27.0
L-methionine	37.5	19.5	10.5	4.0	19.0	20.0	26.5
L-phenylalanine	37.5	27.5	23.0	8.5	21.5	22.0	28.0
L-aspartic	37.5	19.0	22.5	15.5	18.0	17.5	14.5
L-glutamic	37.5	28.0	27.5	20.5	21.5	20.5	23.5
L-tyrosine	37.5	30.0	26.5	11.5	23.5	23.0	34.0
L-lysine	37.5	25.0	24.0	10.5	19.0	19.5	32.5

* No additional amino acids were detected in the media at the end of the period.

TABLE III

Net change in content of 12 amino acids in media after 6 hours exposure to specimens (Initial concentration = 0.05 mM/l; Volume, 750 ml)*

	Initial quantity	Quantity present after 6 hours					
Specimen wet. wt.		*Pisaster* 425 g	*Pisaster* 311 g	*Pisaster* 300 g	*Mediaster* 52 g	*Solaster* 138 g	*Pycnopodia* 94 g
Amino acid	(μM)	(μM)	(μM)	(μM)	(μM)	(μM)	(μM)
L-alanine	37.5	3.2	11.5	11.5	29.0	3.5	3.5
L-valine	37.5	1.0	7.5	9.5	28.0	34.5	2.5
glycine	0.0	6.5	9.5	16.0	1.5	1.0	18.0
L-proline	37.5	8.0	20.0	24.5	36.0	32.0	8.0
L-threonine	37.5	7.0	22.0	21.0	34.5	35.0	8.5
L-serine	37.5	3.5	16.0	17.5	37.5	15.5	5.5
L-methionine	0.0	0.0	0.0	0.0	0.0	0.0	0.0
L-phenylalanine	37.5	1.5	11.5	13.0	35.5	29.0	25.0
L-aspartic	37.5	25.0	36.5	41.0	40.0	36.5	31.5
L-glutamic	37.5	29.0	40.0	37.0	42.0	34.5	35.5
L-tyrosine	37.5	4.5	18.5	19.5	39.5	33.0	5.0
L-lysine	37.5	4.5	17.0	15.5	38.5	39.0	6.0

* No additional amino acids were detected in the media at the end of the periods.

would be uptake or release to these compounds. In the first part of this series, 12 amino acids were included and the results are shown in Table II. While in every case there was a considerable net uptake of amino acids, in two of the experiments with *Pteraster* a net increase in glycine was detected in the media. In the second part of this series, glycine and L-methionine were omitted from the initial mixture (Table III). While *l*-methionine did not subsequently turn up in the media, significant quantities of glycine did. The overall effect again, however, was a very considerable net uptake of amino acids, even though in some instances the concentrations of a few slightly increased. It was further noted that in this series there was less uptake of L-alanine than when it was present alone in the first series. This result was expected on the basis of previously observed competitive inhibition phenomena in the amino acid transport system of starfish (Ferguson, 1968b).

TABLE IV

Net release of amino acids into media in the presence of 1.0 mM/l initial concentration of L-alanine (Volume = 750 ml; time = 6 hours)

Specimen	Wet. wt. (g)	Initial ALA (μM)	Final**										
			ALA (μM)	GLY (μM)	THR (μM)	SER (μM)	CYSH (μM)	MET (μM)	PHE (μM)	ASP (μM)	GLU (μM)	TYR (μM)	LYS (μM)
Pteraster	158	750	413	28.0	—	—	—	—	—	—	—	—	—
Pteraster	132	750	499	35.5	—	—	—	—	—	—	0.5	—	—
Pteraster	220	750	83	18.5	—	—	1.5	—	—	—	—	—	—
*Mediaster**	48	300	273	0.5	—	—	—	—	—	—	—	—	0.5
Patiria	175	750	624	4.5	—	—	—	—	—	—	—	—	—
Patiria	165	750	634	5.0	0.5	—	—	—	—	—	—	0.5	—
Pisaster	402	750	339	120.5	—	5.0	—	—	—	—	1.0	—	—
Pisaster	318	750	88	97.0	—	4.5	—	—	—	—	—	—	—
Pisaster	238	750	431	61.5	—	2.0	—	—	—	—	—	—	—
Solaster	130	750	491	13.0	0.5	—	—	0.5	—	—	—	—	—
Pycnopodia	180	750	167	132.0	0.5	3.0	0.5	—	0.5	0.5	0.5	—	—
*Henricia**	24	300	283	0.5	—	0.5	—	—	—	—	—	—	—

* Volume = 300 ml.

** Only amino acids observed in measurable concentrations are listed.

As the increased net release of glycine and the reduced L-alanine uptake observed in the second series of experiments was probably the result of competitive inhibition, a third series of experiments was carried out to see if net release could be enhanced utilizing this effect. In this series, a relatively high concentration (1.0 mM/l) of L-alanine was used to partially block reabsorption and thus more clearly reveal what amino acids (in the same transport series) are most involved in the release process. As may be seen in Table IV, glycine was by far the most significant amino acid released, although others also turned up. In most cases the net rate of uptake of L-alanine was at least an order of magnitude greater than the net total rate of release of the other amino acids.

While the same general results were obtained with all of the species used, some differences between them may be noticed in the data tables. Most of

these differences are easily explained by the nature of the specimens themselves. Small types, such as *Mediaster* and *Henricia* took up considerably less of the dissolved amino acids than the larger species. Species with large surface areas, such as *Pycnopodia* and *Solaster*, were relatively efficient in their uptake in spite of the modest size of the specimens employed. *Pteraster* also took up amino acids very rapidly, but this species possess a unique aboral chamber which it actively ventilates.

Discussion

In all the cases examined in this study, the net flux of amino acids was overwhelmingly inward, as one would expect it to be if these animals derive net benefit from dissolved environmental nutrients. The quantity taken up was considerably larger when the starfishes were exposed to greater concentrations, but a higher proportion was taken up when lower concentrations were employed. This result is compatible with those of previous studies of starfish transport mechanisms (Ferguson, 1964, 1968b).

It must be noted, however, that the least concentration used, 0.05 mM/l L-alanine, while very low in absolute terms, was still quite a bit higher than that found in "natural" sea water. This condition is evident in the fact that clean natural sea water was used in the formulation of the media used, and it did not contain sufficient concentrations of amino acids to be significantly registered by the analytical techniques employed. It would seem, though, that concentrations such as those used could easily occur in the microenvironments of the animals, particularly during feeding activities—but experimental verification of this is as yet lacking.

In spite of these practical difficulties, the experimental situation employed does provide considerable insight into the nutritional processes. The net inward flux is so great even at the lowest concentrations used, that this effect could certainly be extrapolated to sea water much less rich in nutrients. Furthermore, probably as a result of the relief of the competitive inhibition of the transport system, net release of amino acids appears to become more limited as there is a decrease in the concentration in the media of amino acids of the same transport group. Thus, the present results definitely support the concept that starfish can receive net benefit from the dissolved nutrients that may be found in their environment.

There is no question, however, that these animals are somewhat "leaky" systems. When the transport mechanism is inhibited they lose the ability to retain their natural pools. It would appear, then, that the transport system has as equal importance in retaining endogenous amino acids as in taking up exogenous ones. Any factor influencing the transport system, such as reduced salinity, temperature, inhibiting compounds in the sea water, *etc.*, would be expected to influence the composition of the internal amino acid pools, and thus profoundly effect the physiological state of the animals. Previously, this consequence has not always been fully appreciated.

The specific amino acids released, the primary one being glycine, seem to be those that predominate in the tissue pools. Preliminary analyses of the free amino acids found in the body walls of these species indicate that glycine is often present at over 100 times the concentrations of the other major amino acid components.

This high glycine concentration has also been noted in the few published accounts of the free amino acid content of other starfishes (*e.g.*, Giordano, Harper and Filice 1950; Jeuniaux, Bricteux-Grégoire and Florkin, 1962). The significant amounts of *l*-serine and other amino acids that turned up in the media of the third series of experiments also appear to correlate with the presence of these compounds in the tissue pools of the various species, but a more comprehensive study of the free amino acids of these species has not yet been completed.

It is becoming increasingly evident, from the results of this study and others in the literature, that the free amino acid pools maintained in the various tissues of these animals are in a delicate balance with chemical and physical factors of the external environment and, doubtless, the internal physiological state. Practically nothing is yet known about the interaction of these parameters in the microenvironments of the various species throughout the seasons of the year or the life history of the animals. Obviously this whole area must be explored in future research if a true picture of the biology of these animals is to be developed.

Summary

1. Net uptake and release of dissolved free amino acids was measured in experiments with 10 species of Puget Sound starfishes, utilizing gas liquid chromatography.

2. When specimens were placed in filtered sea water with 0.5 mM/l L-alanine, most of the amino acid was removed within a 6 hour period, and no more than trace quantities of other amino acids appeared in the media.

3. When specimens were placed in filtered sea water with 0.5 mM/l concentrations of 12 amino acids, there was considerable net uptake of all the amino acids except glycine, which in two cases with *Pteraster* was further released into the media.

4. In a similar experiment in which glycine and L-methionine were omitted from the mixture, there was significant net release of glycine but not *l*-methionine, simultaneously with considerable uptake of the majority of the other 10 amino acids in most cases.

5. When the uptake mechanism for neutral amino acids was partially inhibited by including 1.0 mM/l L-alanine in the media, a fairly large efflux of glycine and occasionally a much lesser amount of *l*-serine and other amino acids was detected. The amino acids released by the various species appeared to correlate with those maintained free in their tissues.

6. The results of the study support the concept that starfish can receive net benefit from dissolved nutrients in their environments. They further indicate that the transport system for taking up amino acids is also significant in the retention of those amino acids already in the metabolic pools.

LITERATURE CITED

Chau, U. K., and J. P. Riley, 1966. The determination of amino acids in sea water. *Deep Sea Res.*, **13**: 1115–1124.

Ferguson, J. C., 1963. An autoradiographic study of the distribution of ingested nutrients in the starfish, *Asterias forbesi*. *Amer. Zool.*, **3**: 524.

Ferguson, J. C., 1964. Nutrient transport in starfish. II. Uptake of nutrients by isolated organs. *Biol. Bull.*, **126**: 391–406.

Ferguson, J. C., 1967a. Utilization of dissolved exogenous nutrients by the starfishes, *Asterias forbesi* and *Henricia sanguinolenta*. *Biol. Bull.*, **132**: 161–173.

Ferguson, J. C., 1967b. An autoradiographic study of the utilization of free exogenous amino acids by starfishes. *Biol. Bull.*, **133**: 317–329.

Ferguson, J. C., 1968a. An autoradiographic analysis of the uptake of exogenous glucose by three species of starfishes. *Amer. Zool.*, **8**: 805.

Ferguson, J. C., 1968b. Transport of amino acids by starfish digestive glands. *Comp. Biochem. Physiol.*, **24**: 921–931.

Ferguson, J. C., 1969. Feeding activity in *Echinaster* and its induction with dissolved nutrients. *Biol. Bull.*, **136**: 374–384.

Ferguson, J. C., 1970. An autoradiographic study of the translocation and utilization of amino acids by starfish. *Biol. Bull.*, **138**: 14–25.

Giordano, M. B., H. A. Harper and F. P. Filice, 1950. The amino acids of a starfish and a sea urchin (Asteroidea and Echinoidea). *Wasmann. J. Biol.*, **8**: 129–132.

Jeuniaux, C., S. Bricteux-Grégoire and M. Florkin, 1962. Régulation osmotique intracellulaire chez *Asterias rubens*. L. Rôle du glycocolle et de la taurine. *Cah. Biol. Mar.*, **3**: 107–113.

Johannes, R. R., S. J. Coward and K. L. Webb, 1969. Are dissolved amino acids an energy source for marine invertebrates? *Comp. Biochem. Physiol.*, **29**: 283–288.

Roach, D., and C. W. Gehrke, 1969. Direct esterification of the protein amino acids. GLC of N-TFA n-butyl esters. *J. Chromatogr.*, **44**: 269–278.

Siegel, A., and E. T. Degens, 1966. Concentration of dissolved amino acids from saline waters by ligand-exchange chromatography. *Science*, **151**: 1098–1101.

Stephens, G. C., 1962. Uptake of organic material by aquatic invertebrates. I. Uptake of glucose by the solitary coral, *Fungia scutaria*. *Biol. Bull.*, **123**: 648–659.

Stephens, G. C., 1963. Uptake of organic material by aquatic invertebrates. II. Accumulation of amino acids by the bamboo worm, *Clymenella torquata*. *Comp. Biochem. Physiol.*, **10**: 191–202.

Stephens, G. C., 1964. Uptake of organic material by aquatic invertebrates. III. Uptake of glycine by brackish water annelids. *Biol. Bull.*, **126**: 150–162.

Stephen, G. C., and R. A. Schinske, 1961. Uptake of amino acids by marine invertebrates. *Limnol. Oceanogr.*, **6**: 175–181.

Stephens, G. C., and R. A. Virkar, 1966. Uptake of organic material by aquatic invertebrates. IV. The influence of salinity on the uptake of amino acids by the brittle star, *Ophiactis arenosa*. *Biol. Bull.*, **131**: 172–185.

Virkar, R. A., 1966. The role of free amino acids in the adaptation to reduced salinity in the sipunculid *Golfingia gouldii*. *Comp. Biochem. Physiol.*, **18**: 617–626.

Webb, K. L., and L. Wood, 1967. Improved techniques for analysis of free amino acids in sea water. Pages 440–444 in *Automation in Analytical Chemistry, Technicon Symposia, 1966*. Mediad, Inc., White Plains, New York.